HEI 4 156264 16/6/92 D10

English Translation
of Certified Copy

11

04.10.93

PATENT OFFICE

JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application: June 16, 1992 *

Application Number : Patent Application No. Hei 4-156264

Applicant(s) : SANKYO COMPANY, LIMITED

Date : June 4, 1993

Commissioner, Wataru Aso
Patent Office

Pri

Official Seal

Certificate Serial No. Hei 05-027009

IN THE MATTER of an application
for letters patent under
The European Patent Convention

04.10.93

I, Takashi Taniyama, who am a Deputy Director of Patent Department of SANKYO COMPANY, LIMITED of 2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo, Japan, translator of the attached document reference No.Hei 04-156264 and fully conversant with the English and Japanese languages, do hereby certify that to the best of my knowledge and belief the following is a true translation into the English language of the accompanying certified copy of documents filed in the Japanese Patent Office on the June 16, 1992 in respect of an application for Letters Patent including a translation of the Official Certificate accompanying the said copy.

Signed this 31th day of August, 1993

Takashi Taniyama

04-156264

Name of Document	Patent Application
Docket Number	920185N 04.10.92
Filing Date	June 16, 1992
Address To:	Commissioner, Patent Office
International Patent Classification	C07K 15/04
Title of Invention	Phospholipase A1
Number of Claim	3
Inventor	
Address or Domicile	SANKYO COMPANY, LIMITED 2-58, Hiromachi 1-chome , Shinagawa-ku, Tokyo 140 Japan
Name	Atsushi Hattori
Inventor	
Address or Domicile	SANKYO COMPANY, LIMITED 2-58, Hiromachi 1-chome , Shinagawa-ku, Tokyo 140 Japan
Name	Noriyoshi Uchida
Inventor	
Address or Domicile	SANKYO COMPANY, LIMITED 2-58, Hiromachi 1-chome , Shinagawa-ku, Tokyo 140 Japan
Name	Masahiro Kitaoka
Patent Applicant	
Identification Number	000001856
Address or Domicile	5-1, Nihonbashi Honcho 3-chome, Chuo-ku, Tokyo
Name	SANKYO COMPANY, LIMITED
Representative	Yoshibumi Kawamura
Agent	
Identification Number	100081400
Address or Domicile	c/o SANKYO COMPANY, LIMITED 2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo
Patent Attorney	
Name	Akio Ohno

[NAME OF DOCUMENT] SPECIFICATION

[TITLE] Phospholipase A1

04.10.93

[SCOPE OF CLAIM FOR PATENT]

[Claim 1]

Phospholipase A1 that selectively hydrolyzes an acyl group at the 1-position of a phospholipid and has the action of producing the 2-acyl form of lysophospholipid, an optimum active pH of 3.5 to 4.5, a stable pH region of 5.5 to 10.5, and a molecular weight of 37,000 and an isoelectric point of pI 3.9, or a molecular weight of 35,000 and an isoelectric point of pI 4.3.

[Claim 2]

The phospholipase A1 according to Claim 1 produced by the fungus of the Aspergillus species.

[Claim 3]

The phospholipase A1 according to Claim 1 produced by Aspergillus oryzae.

[DETAILED DESCRIPTION OF THE INVENTION]

[Object of the Invention]

[Field of Utilization in Industry]

The present invention relates to a phospholipase A1 that selectively hydrolyzes an acyl group at the 1-position of a phospholipid, and produces the 2-acyl form of lysophospholipid, and more particularly, to a phospholipase A1 produced by the fungus of the Aspergillus species.

[Prior Art]

Lecithin is a phospholipid having surface activating action, antioxidation action, physiological activating action and so forth, that is widely used in the food industry, feed products and pharmaceutical products. However, the surface activating action of lecithin is inferior in comparison with other surface active agents used for food product in terms of emulsification stability. Thus, research has been conducted to improve its emulsification stability using enzymatic hydrolysis.

Lysophospholipids are partially decomposed phospholipids that are obtained by hydrolyzing a portion of fatty acid that is ester-bonded to a glycerine residue of a phospholipid. It is reported that since lysophospholipids have enhanced emulsification properties as well as a broad temperature range over which emulsification strength is demonstrated due to their increased water-solubility compared with phospholipids, lysophospholipids are imparted with properties that demonstrate emulsification strength even when added to either oil or aqueous systems, do not suffer reduced emulsification strength even in the presence of metal ions such as calcium and magnesium, and demonstrate favorable emulsification stability under the acidic condition.

Lysophospholipids obtained by decomposition of

phospholipid using enzymes are presently commercially available. These commercial products use phospholipase A2 activity contained in the enzyme preparation, pancreatin, prepared from porcine pancreas. These commercial products had disadvantages in terms of having the characteristic odor of animal organs and having limitations on the amount able to be supplied, resulting in expensive costs. Thus, an alternative to pancreatin has been sought after as a source of phospholipase A.

In addition, several methods involving decomposition of phospholipid using phospholipase A (Japanese Unexamined Patent Publication No. Sho-58-212783) and lipases (Japanese Unexamined Patent Publications No. Sho-63-42691) produced by microorganisms have previously been disclosed. In addition, it has long been known that lipase having phospholipid decomposing activity exists in Taka-Diastase (registered trademark) originating in Aspergillus oryzae which belongs to Aspergillus species, the so-called "enzyme treasure chest" (Biochem. Z., 261, 275 (1933)). However, the enzyme activity resulting from methods using these enzymes is lower than that of pancreatin, or in the case of using enzymes having inferior substrate specificity, additional disadvantages were encountered in terms of poor yield of lysophospholipid or the risk of causing lowered quality of the lysophospholipid due to the presence of byproducts. Thus, the development of an enzyme that can be supplied

both reliably and at low cost, while also having superior activity and selectivity, has been desired in order to overcome the above-mentioned problems and disadvantages.

[Problems to be Solved by the Invention]

The present inventors have made intensive studies on microorganisms that produce phospholipase as well as that phospholipase for an extended period of time, and found highly purified phospholipase A1 having both superior activity and selectivity by isolation and purification of enzyme produced by the fungus of the Aspergillus species, to accomplish the present invention.

[Constitution of the Invention]

[Means for Solving the Problems]

The phospholipase A1 of the present invention consists of an enzyme protein (phospholipase A1a), having a molecular weight of 37,000 and an isoelectric point of pI 3.9, as well as an enzyme protein (phospholipase A1b), having a molecular weight of 35,000 and an isoelectric point of pI 4.3, both of which have the characteristics described below.

1. Action and Substrate Specificity

The present enzyme hydrolyzes only the acyl group at the 1-position of a phospholipid, and its form of action belongs to that of phospholipase A1 (enzyme number:

EC3.1.1.32).

04.10.93

2. Optimum pH

The optimum pH of the present enzyme is from 3.5 to 4.5 using an ordinary activity measuring system (reacting at 37°C for 10 minutes in the presence of Triton X-100), and the maximum activity is obtained at approximately pH 4 (see Fig. 1). In addition, the optimum pH is from 4.5 to 5.5 when using an activity measuring system (reacting at 37°C for 10 minutes) containing no Triton X-100 (see Fig. 2).

3. Stable pH Region

The stable pH region of the present enzyme varies due to the effects of the concentration of the enzyme itself, as well as the types and concentrations, etc., of other substances present. More specifically, the stable pH region of an approximately 10 units/ml enzyme solution is 5.5 or more in a 33 mM acetic acid/sodium acetate buffer solution, and 10.5 or less in a 33 mM glycine/sodium chloride - sodium hydroxide buffer solution (see Fig. 3).

4. Measurement of Titer

Phospholipase Activity Test

To 0.5 ml of an aqueous solution of 2.0 (w/v)% of SLP-White (manufactured by True Lecithin Kogyo Co., Ltd.) and 4 (v/v)% Triton X-100 are added 0.05 ml of 0.1 M

calcium chloride and 0.25 ml of 0.2 M acetate buffer solution (pH 4.0). Next, 0.1 ml of the enzyme solution is added to the resulting mixture followed by stirring to obtain a homogeneous mixture and allowing to stand at 37°C while carrying out the enzyme reaction for 10 minutes. Subsequently, 0.1 ml of 1 N hydrochloric acid is added to stop the enzyme reaction. An amount of 0.02 ml of the reaction mixture is sampled to determine the amount of free fatty acid using a free fatty acid quantitative reagent, Determiner NEFA (manufactured by Kyowa Medex Co., Ltd.). The enzyme activity that produces 1 mol of fatty acid per minute of the enzyme reaction is taken to be 1 unit.

5. Active Optimum Temperature Range

The present enzyme has activity from 30°C to 65°C, and the optimum temperature is 50°C to 60°C, with the maximum enzyme activity demonstrated in the vicinity of 55°C (see Fig. 4).

6. Inhibition, Activation and Stabilization

The present enzyme is not observed to be particularly inhibited by polyvalent metal ions such as mercury, lead and iron. In addition, there are also no changes observed in activity due to the presence of ethylenediamine-tetraacetic acid (EDTA).

7. Purification Method

Cooled acetone is added to a final concentration of 60 to 80 (v/v)% while stirring to a an extract solution of Koji culture (or a solid culture on wheat bran) extract of the fungus of the Aspergillus species. After the resulting mixture is left to stand for a while, it is centrifuged to obtain the precipitate. In addition, a similar preciptate is obtained even if ethanol is used instead of acetone. This precipitate is dissolved in 50 mM acetate buffer solution (pH 5.5) followed by salting-out with ammonium sulfate and centrifugation to separate the precipitate. After the resulting precipitate is dissolved in 50 mM acetate buffer (pH 5.5) containing 1 M ammonium sulfate and insolubles are filtered out, the residue is purified using column chromatography (column: Butyl-Toyopearl Pak 650S, manufactured by Tosoh Corp.; effluent solvent: 50 mM acetate buffer solution containing ammonium sulfate). After the resulting semi-purified enzyme is dialyzed against 20 mM acetate buffer solution (pH 5.5), the resulting product is again purified using column chromatography (column: Q-Sepharose, manufactured by Pharmacia AB: effluent solvent: 20 mM acetate buffer solution (pH 5.5) containing sodium chloride) to obtain phospholipase A1. Moreover, after the thus obtained phospholipase A1 is subjected to salting-out with ammonium sulfate and gel filtration (column; Superose 12, manufactured by Pharmacia AB), the resulting product is purified using column chromatography (column; MonoQ,

manufactured by Pharmacia AB; effluent solvent: 20 mM acetate buffer solution (pH 4.5)) containing sodium chloride) to obtain enzyme proteins phospholipase Ala and Alb.

9. Molecular Weight

The molecular weight of the present enzyme is measured by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [K. Weber, et al., J. Biol. Chem., 244, 4406 (1969)]. The values for phospholipase Ala and Alb are 37,000 and 35,000, respectively. In addition, the isoelectric point of the present enzyme is measured by isoelectric point electrophoresis. The values for phospholipase Ala and Alb are pI 3.9 and pI 4.3, respectively.

10. Crystal Structure

Since the present enzyme is not obtained in the form of a crystal, its crystal structure cannot be determined.

Examples of productive microorganisms that produce acidic phospholipase Al include the fungi belonging to the species Aspergillus such as Aspergillus oryzae (e.g., SANK-11870, IFO-30102), Aspergillus niger (e.g., IFO-4407), Aspergillus usamii (e.g., IFO-6082), and Aspergillus awamori (e.g., IFO-4033), and preferably Aspergillus oryzae. Each of these fungi are known microorganisms (as indicated in, for example, Japanese

Patent Publication No. Sho-46-32792, J. Gen. Appl. Microbiol., 17, 281 (1971) and Biochem. Z., 261, 275 (1933)). The respective numbers of these fungi are stored in storage institutions such as the Institute for Fermentation - Osaka (IFO), and they can be freely furnished with. In addition, SANK-11870 has been deposited in the Fermentation Research Institute of the Agency of Industrial Science and Technology as fermentation research institute deposition No. 3887 (FERM BP-3887). In addition, these strains include their original natural strains as well as their artificial variant strains.

Culturing of the strain is performed using wheat bran culturing technique by inoculating a solid medium, obtained by adding 0.5- to 2-fold weight (and preferably an equivalent amount) of water to wheat bran bread followed by boiling, with the seed bacteria. In addition to wheat bran, grain (e.g., rice, wheat), various miscellaneous grains (e.g., corn, soybeans, sesame seeds) and cottonseed cake can be used alone or in combination for the raw materials of the medium.

The culturing temperature is 10°C to 40°C (and preferably 20°C to 32°C), and while the culturing time varies depending on medium composition, culturing temperature and so forth, it is usually 3 days to 20 days (and preferably 4 days to 8 days).

After completion of culturing, 1- to 20-fold weight of

water or a suitable buffer solution (for example, acetate buffer solution or phosphate buffer solution) is added to the koji and the resulting mixture is stirred well followed by compression filtration to obtain the enzyme solution.

Moreover, the enzyme of the present invention can be collected from the above-mentioned enzyme solution, and purified by isolation using conventional techniques such as salting-out technique, organic solvent precipitation, adsorption technique using an ion exchanger for the adsorbent, ultrafiltration or vacuum drying either alone or in a suitable combination.

[Effect of the Invention]

Phospholipase A1 of the present invention is a extremely useful phospholipase, which is a microorganism-derived enzyme having excellent activity and selectivity, and can be supplied both reliably and inexpensively.

The enzyme reaction for decomposing phospholipid using the present enzyme is carried out by bringing enzyme and substrate in contact either in an aqueous medium or in the wet state.

Examples of substrates used include lecithins of wheat flour, soybeans or egg yolk, and that concentration is preferably 1 to 50 % by weight.

While well water or tap water can be used as such for

the water employable, water can be used to improve the efficiency of the enzyme reaction by adding acid (e.g., acetic acid), alkali (e.g., sodium hydroxide) or buffer solution (e.g., acetate buffer solution) to adjust the pH to a value of 3 to 7 (and particularly preferably from 3.5 to 5.5).

The reaction temperature is from 10°C to 70°C (preferably 20°C to 65°C, and particularly preferably about 30°C to 60°C), and while the time required for the reaction varies depending on the substrate, reaction temperature, pH, etc., it is usually from 10 minutes to 10 days (preferably 1 hour to 2 days).

After completion of the enzyme reaction, the resulting lysophospholipid can be used directly in processing treatment without separation. Alternatively, insolubles are filtered out using ordinary techniques and the residue is used as such or concentrated to give a suitable lysophospholipid solution. Moreover, the resulting lysophospholipid can also be obtained by adding water, extracting with a water-immiscible organic solvent and removing the extraction solvent, as necessary. In addition, the resulting lysophospholipid can also be further purified, as necessary, using ordinary techniques such as column chromatography and recrystallization.

The present invention is described below more specifically by way of Examples, but it is not limited thereto.

[Example]

Example 1

Crude Phospholipase A

The SANK 11870 strain of Aspergillus oryzae was cultured at 30°C for 6 days in 12 g of media consisting of a mixture of equal amounts of wheat bran and water. Next, all of the precultured strain were inoculated in a medium in which 600 g of a mixture of wheat bran and water had been placed in a metal dish (42 x 24 x 7 (depth) cm) and boiled at 120°C for 30 minutes. The strain was then cultured at 30°C for 15 hours and further at 19°C for 5 days. To the thus prepared koji was added 3 liters of water, followed by mixing well. After allowing to stand at 37°C for 2 hours, the mixture was filtered to obtain 2.87 liters of enzyme extract of phospholipase A with a titer of 5.9 units/ml. Acetic acid was added to this enzyme extract to adjust the pH to 4.0, 3-fold amount of cold acetone was added thereto, and the mixture was allowed to stand overnight in a cold room. The supernatant was then discarded and the precipitate was sufficiently washed with acetone and dried in vacuo to obtain 11.1 g of a crude powder of phospholipase A1 with a titer of 1,173 units/g.

Example 2

Phospholipase A1a and A1b

In approximately 100 ml of water was dissolved 10 g of the crude phospholipase A1 obtained in Example 1, and 1 N acetic acid was added to the resulting solution to adjust to pH 4.0. To the mixture was added water to make the volume of the mixture 200 ml, and 200 ml of cold acetone was admixed with the mixture followed by allowing to stand for 1 hour. Subsequently, the mixture was centrifuged to obtain the first precipitate. This precipitate had amylase activity, partial protease activity and phospholipase A activity of 320 units. To the supernatant was then added 600 ml of cold acetone followed by mixing and allowing to stand for 1 hour. Thereafter, the mixture was centrifuged to obtain the second precipitate. This precipitate had phospholipase A activity of 7,800 units. The second precipiate was dissolved in 500 ml of 50 mM acetate buffer solution (pH 5.5), and 300 g of ammonium sulfate was added thereto, followed by salting-out. After the precipitate obtained by centrifugation was dissolved in 50 mM acetate buffer solution containing 1M ammonium sulfate (pH 5.5) and insolubles were removed by filtration, column chromatography was performed (column: Butyl Toyopearl Pak 650S, manufactured by Tosoh Corp.; effluent solvent: 50 mM acetate buffer containing ammonium sulfate (pH 5.5); ammonium sulfate concentration: 1 to 0 M; gradient elution). The phospholipase A activity fraction not containing amylase and protease was eluted with ammonium sulfate at a concentration of 0.6 M or

less. After dialyzing this phospholipase A activity fraction with 20 mM acetate buffer solution (pH 5.5), the resulting product was purified using column chromatography (column: Q-Sepharose, manufactured by Pharmacia AB; effluent solvent: 20 mM acetate buffer containing sodium chloride; salt concentration: 0 to 0.5 M; gradient elution) to obtain 4,000 units of phospholipase A1 fraction. The phospholipase A1 was then allowed to act on L-α-di-palmitoyl-phosphatidylcholine (NEN, NEC-764), wherein a palmitoyl group at the 2-position is labelled with ^{14}C, and L-α-di-palmitoyl-phosphatidylcholine (NEN, NEC-682), wherein the palmitoyl groups at the 1- and 2-positions are labelled with ^{14}C. As a result, ^{14}C-labelled plamitic acid was only liberated from phosphatidylcholine wherein plamitoyl groups at the 1- and 2-positions were labelled with ^{14}C, thus confirming that this enzyme acts on 1-acyl groups only.

To the above-mentioned phospholipase A1 was added ammonium sulfate to effect salting-out, and the resulting product was subjected to gel filtration (column: Superose 12, manufactured by Pharmacia AB; effluent solvent: 20 mM acetate buffer (pH 4.5)) and then column chromatography (column: MonoQ, manufactured by Pharmacia AB; effluent solvent: 20 mM acetate buffer solution containing sodium chloride (pH 4.5)); salt concentration: 0 to 0.25 M; gradient elution) to obtain the desired substances in the form of 880 units of phospholipase A1a and 2,400 units of

phospholipase A1b.

04.10.97

Phospholipase A1a

Molecular weight: 37,000 (as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate).

Isoelectric point: pI 3.9 (as determined by isoelectric point electrophoresis).

Optimum active pH: pH 3.5 to 4.5 (in the presence of Triton X-100) or pH 4.5 to 5.5 (in the absence of Triton X-100) (the relationship between pH and enzyme activity (pH-activity curve) is indicated in Fig. 1 (in the presence of Triton X-100) and Fig. 2 (in the absence of Triton X-100)).

Stable pH region: pH 5.5 or higher (with respect to an approximately 10 units/ml enzyme solution in 33 mM acetic acid/sodium acetate buffer solution) or pH 10.5 or less (with respect to an approximately 10 units/ml enzyme solution in 33 mM glycine/sodium chloride - sodium hydroxide buffer solution) (the relationship between residual enzyme activity following pre-treatment of the enzyme at 37°C for 1 hour and pre-treatment pH is indicated in Fig. 3 (enzyme pH stability; pH 3.2-6.0: acetic acid/sodium acetate buffer solution, pH 5.5-8.5: potassium monophosphate/sodium diphosphate buffer solution, pH 8.0-12.5: glycine/sodium chloride - sodium hydroxide buffer solution)).

Optimum temperature: 50°C to 60°C (the relationship between temperature and enzyme activity (temperature-activity curve) is indicated in Fig. 4).

Phospholipase A1b

Molecular weight: 35,000 (as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate).

Isoelectric point: pI 4.3 (as determined by isoelectric point electrophoresis).

Optimum active pH: pH 3.5 to 4.5 (in the presence of Triton X-100) or pH 4.5 to 5.5 (in the absence of Triton X-100) (the relationship between pH and enzyme activity (pH-activity curve) is indicated in Fig. 1 (in the presence of Triton X-100) and Fig. 2 (in the absence of Triton X-100)).

Stable pH region: pH 5.5 or higher (with respect to an approximately 10 units/ml enzyme solution in 33 mM acetic acid/sodium acetate buffer solution) or pH 10.5 or less (with respect to an approximately 10 units/ml enzyme solution in 33 mM glycine/sodium chloride - sodium hydroxide buffer solution) (the relationship between residual enzyme activity following pre-treatment of the enzyme at 37°C for 1 hour and pre-treatment pH is indicated in Fig. 3 (enzyme pH stability; pH 3.2-6.0: acetic acid/sodium acetate buffer solution, pH 5.5-8.5: potassium monophosphate/sodium diphosphate buffer

solution, pH 8.0-12.5: glycine/sodium chloride - sodium hydroxide buffer)).

Optimum temperature: 50°C to 60°C (the relationship between temperature and enzyme activity (temperature-activity curve) is indicated in Fig. 4).

Incidentally, in Figs. 1 to 4, the mark ● indicates the values for phospholipase Ala, while the mark ○ indicates the values for phospholipase Alb.

[BRIEF DESCRIPTION OF THE DRAWINGS]

[Fig. 1]

Fig. 1 indicates the pH-activity curve of the enzyme of the present invention (in the presence of Triton X-100).

[Fig. 2]

Fig. 2 indicates the pH-activity curve of the enzyme of the present invention (in the absence of Triton X-100)

[Fig. 3]

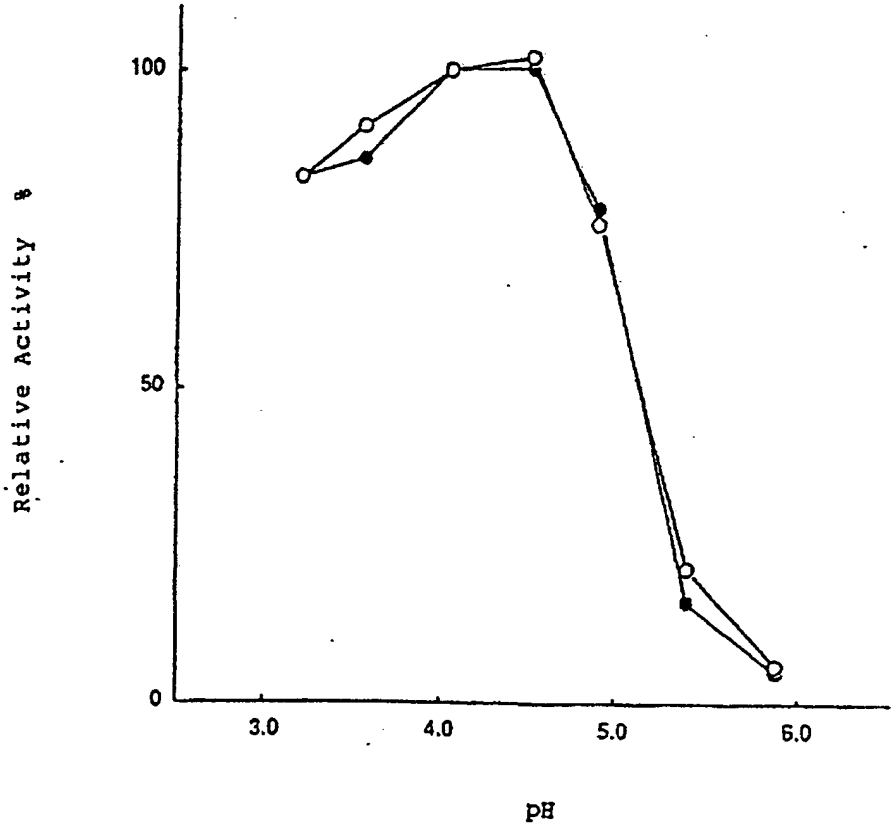
Fig. 3 indicates the pH stability of the enzyme of the present invention (pH 3.2-6.0: acetic acid/sodium acetate buffer solution, pH 5.5-8.5: potassium monophosphate/sodium diphosphate buffer solution, pH 8.0-12.5: glycine/sodium chloride - sodium hydroxide buffer).

[Fig. 4]

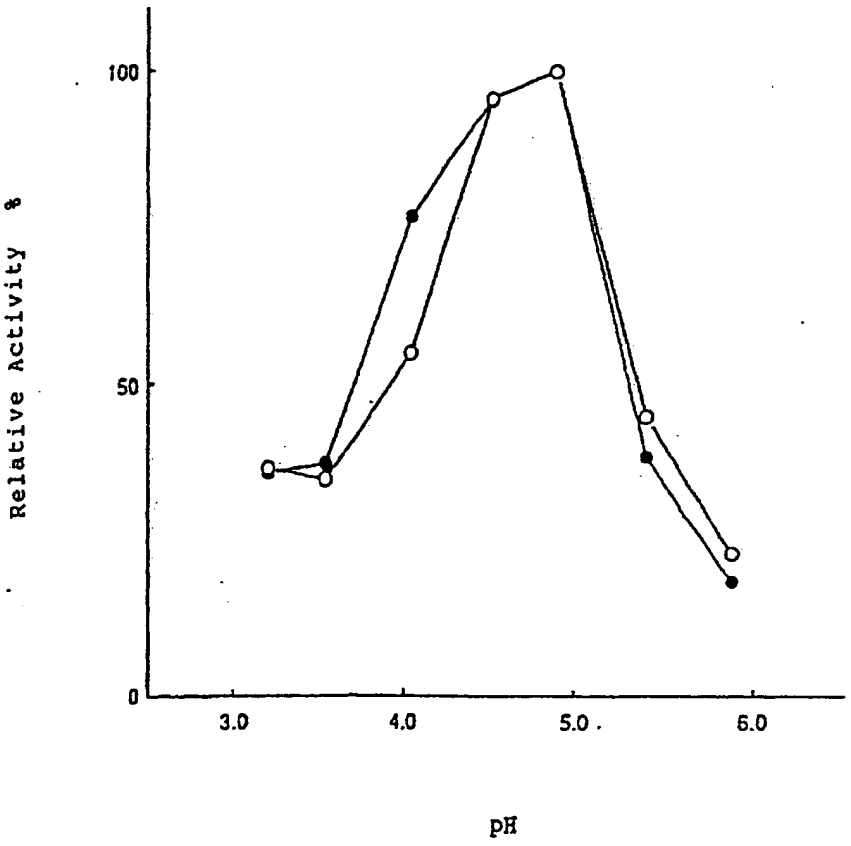
Fig. 4 indicates the temperature-activity curve of the enzyme of the present invention.

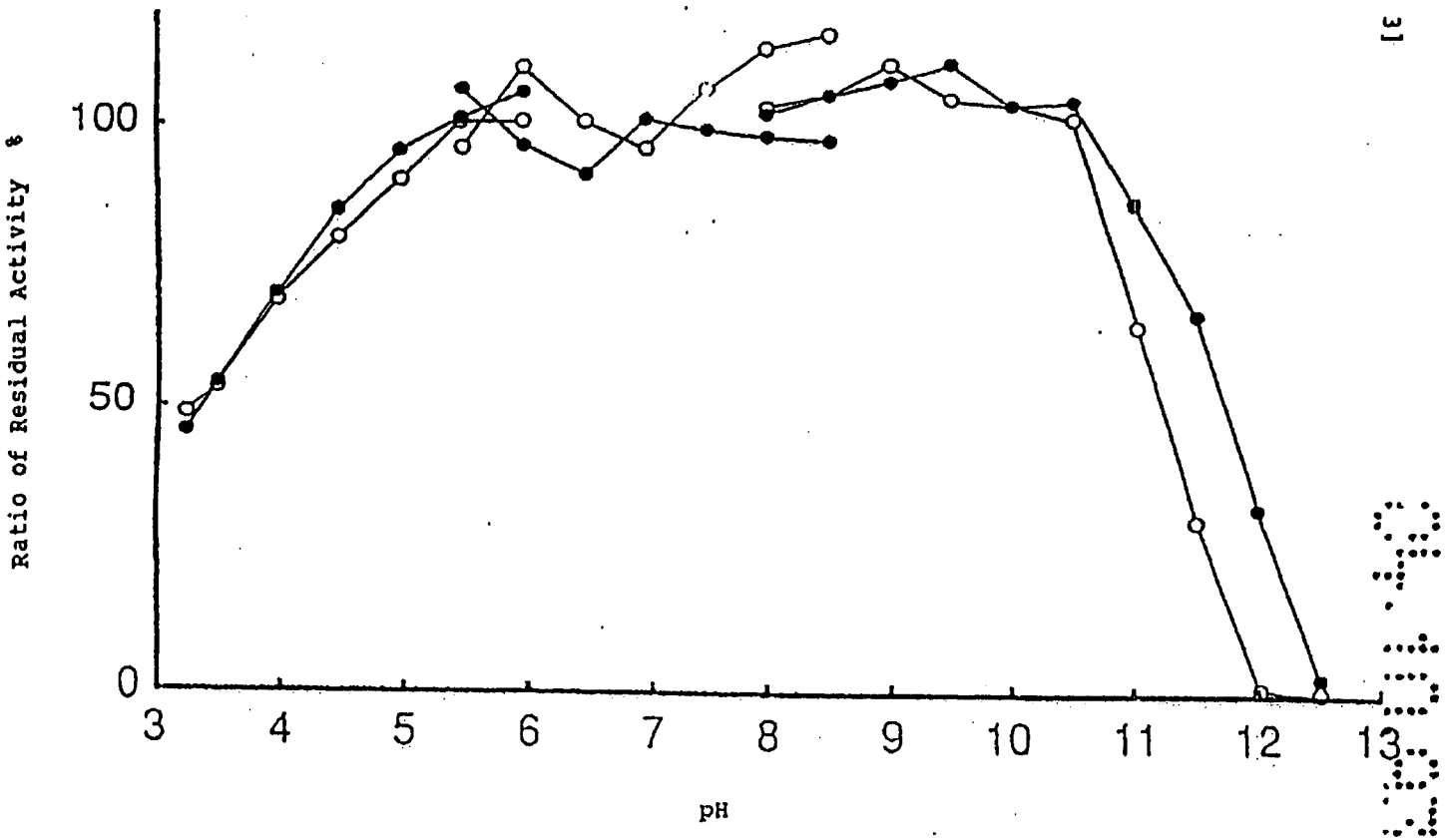
[DOCUMENT NAME] DRAWINGS

[Fig. 1]



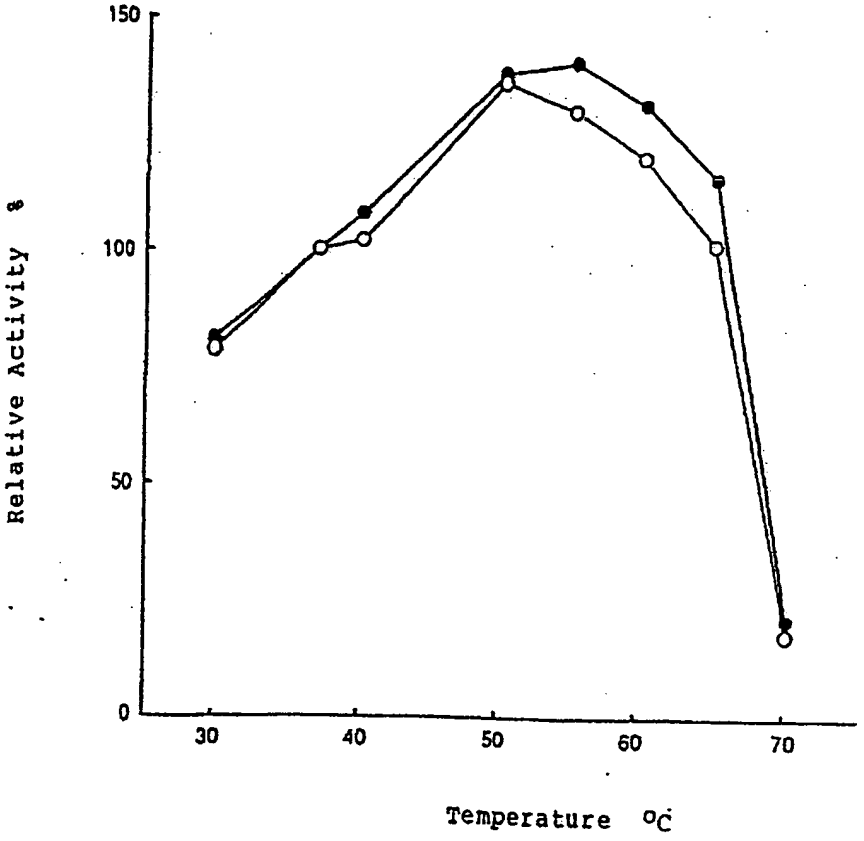
[Fig. 2]





[Fig. 3]

[Fig. 4]



[NAME OF DOCUMENT] ABSTRACT

[ABSTRACT]

[CONSTITUTION] Phospholipase A1 that selectively hydrolyzes an acyl group at the 1-position of a phospholipid and has the action of producing the 2-acyl form of lysophospholipid, an optimum active pH of 3.5 to 4.5, a stable pH region of 5.5 to 10.5, and a molecular weight of 37,000 and an isoelectric point of pI 3.9, or a molecular weight of 35,000 and an isoelectric point of pI 4.3.

[EFFECT] The enzyme of the present invention has superior phospholipase activity as well as superior selectivity.

[SELECTED DRAWING] None

04-156264

Name of Document	Data to be amended by authority
Document to be amended	Patent Application

Recognition information & Addition information

Patent Applicant	
Identification Number	000001856
Address or Domicile	5-1, Nihonbashi Honcho 3-chome, Chuo-ku, Tokyo
Name	SANKYO COMPANY, LIMITED
Agent	Applicant
Identification Number	100081400
Address or Domicile	c/o SANKYO COMPANY, LIMITED Patent Department 2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo
Name	Akio Ohno

04-156264

Information on Applicant's History

Identification Number [000001856]

1. Date of Alteration August 15, 1990
[Reason for Alteration] Newly registered
Address 5-1, Nihonbashi Honcho 3-chome, Chuo-ku, Tokyo
Name SANKYO COMPANY, LIMITED